

Docket No. 46865/59572  
Express Mail Label No. EV342613594US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
NEW PATENT APPLICATION**

**TITLE:** TEST SYSTEM FOR THE DEVELOPMENT OF  
THERAPEUTIC AGENTS, IN PARTICULAR ACTIVE  
COMPOUNDS FOR THE TREATMENT OF  
OSTEOPOROSIS

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**TEST SYSTEM FOR THE DEVELOPMENT OF THERAPEUTIC AGENTS, IN PARTICULAR ACTIVE COMPOUNDS FOR THE TREATMENT OF OSTEOPOROSIS**

The present invention relates to a test system for the identification and testing of 5 active compounds, which act on synaptic transmission (active compounds for treatment of neuronal diseases), which influence endo/exocytosis, which influence processing of proteins and in particular of active compounds which can be used for treatment of osteoporosis or Paget's disease, for treatment of neurological and neuromuscular diseases and other nerve diseases or as psychotropic pharmaceuticals. The invention 10 furthermore relates to a non-human mammal, preferably a rodent, in which one or more chloride channels from the group consisting of CIC-3, CIC-4, CIC-6 and CIC-7 are not expressed or are expressed non-functionally, and to somatic cell lines which are derived, for example, from such an animal, and to the use thereof for the identification and testing 15 of substances which are suitable for influencing in their activity, in particular inhibiting or activating, chloride channels, in particular CIC-3, CIC-4, CIC-5, CIC-6 and/or CIC-7.

Osteoporosis is a disease in which increased degradation of bone occurs, leading to fragility. Osteoporosis is widespread in elderly persons, in particular in elderly women (of hormonal origin). For this reason, sex hormones, which can indeed stop the 20 process of bone degradation, but in most cases have serious undesirable side effects, are often administered to elderly female patients. Specific osteoporosis medicaments have not hitherto been developed.

In the context of the present invention, it has now been found, surprisingly, that mutations in the nucleic acid sequence encoding the CIC-7 protein (chloride channel CIC-7) leads to the expression of a non-functional protein, or completely suppresses 25 expression of such protein thereby causing a very severe form of osteopetrosis in mice. On the basis of these surprising results, it was found that patients with severe juvenile osteopetrosis also have mutations in the CIC-7 gene.

The chloride channel CIC-7 is a predominantly intracellular chloride channel present in late endosomes and lysosomes. CIC-7 is expressed ubiquitously, and in 30 particular also occurs in osteoclasts, the bone-degrading cells. Mutations that lead to the expression of a non-functional CIC-7 protein or that completely suppress expression (called "knock-out" or "KO" in the following), prevent osteoclasts from being able to degrade bone. More detailed studies have shown that CIC-7 is incorporated together with the proton pump into the so-called "ruffled membrane". The ruffled membrane borders 35 the resorption lacunae and acid equivalents are transported into the resorption lacunae via a proton-pump, and the electroneutral transport of HCl into the lacunae is ensured by a parallel chloride conductance carried by CIC-7. An acidic pH in the lacunae is essential for the bone degradation. If an appropriate chloride conductance is absent, the proton

pump cannot pump effectively, with the consequence that osteoclasts cannot acidify the resorption lacunae and cannot destroy the bone.

By knock-out of CIC-7, severe degeneration of the retina furthermore occurs, and neurodegeneration in the central nervous system (CNS) is moreover observed. These 5 observations can be attributed to the fact that the late-endosomal and lysosomal acidification and degradation is impaired in many tissues by CIC-7 knock-out.

In the context of the present invention, it has also been found that chloride channels of the CLC gene family are also involved in the acidification of synaptic vesicles. This has been demonstrated, for example, by knock-out of the CIC-3 channel. 10 With this knock-out, changes in synaptic transmission in the central nervous system and neuronal degeneration occur. It has furthermore been found that other CIC channels, such as CIC-4 and CIC-7, are present in synaptic vesicles. Synaptic vesicles take up neurotransmitters, which are then released into the synaptic gap via exocytosis and, thus, modulate (stimulate or inhibit) the downstream nerve cell. The uptake of 15 neurotransmitters into synaptic vesicles is driven by the pH gradient and the potential gradient across the membrane of the synaptic vesicles (cf. literature references (62)-(67)), so that the activity of chloride channels in synaptic vesicles influences signal transduction in the nervous system.

By the studies set up in the context of the present invention, it is now possible to 20 provide a test system, which enables identification and testing of substances that inhibit one or more chloride channels from the group consisting of CIC-1, CIC-2, CIC-Ka, CIC-Kb, CIC-3, CIC-4, CIC-5, CIC-6 and/or CIC-7 - in particular the predominantly intracellular chloride channels CIC-3, CIC-4, CIC-5, CIC-6 and/or CIC-7 - or of otherwise influencing them in activity, i.e. for example activating them or modifying their regulation. In 25 particular, for the first time a test system and a process are provided for the identification and testing of substances, which (completely or partly) inhibit the chloride channel CIC-7, in particular substances which are suitable for treatment of osteoporosis or Paget's disease. Such a test system also enables identification of substances that influence neuronal signal transduction, and are therefore suitable for treatment of neuronal 30 diseases.

The invention is based on the consideration that a (partial) inhibition of the CIC-7 chloride channel inhibits osteoclast function and, therefore, counteracts bone degradation. Research along the same line is being conducted in the pharmaceutical industry, where effective inhibitors of the proton pump are being searched for. By the 35 present knowledge, it is now possible for the first time to identify substances which act specifically on the CIC-7 chloride channel and cause partial or complete inhibition or activate the channel or modify regulation thereof. A total KO of CIC-7 also influences other tissue and causes e.g. degeneration of the retina and degeneration of the CNS. Therefore pharmacological inhibition of CIC-7 (i.e. by administration of CIC-7 inhibitors)

could potentially have similar effects. This problem can be solved on the one hand by only partial inhibition of the channel, and on the other hand by using active compounds or pharmaceuticals which do not reach undesirable target organs (i.e. eye and brain) (e.g. because of the blood-brain barrier). Further, as CIC-7 exerts its role in bone degradation 5 in the plasma membrane of osteoclasts, but is in intracellular vesicles in neurons, drugs may be designed that do not enter cells and therefore act specifically on osteoclast-expressed CIC-7.

The invention is furthermore based on the assumption that synaptic transmission in the nervous system can be influenced by an inhibition or stimulation of chloride 10 channels. Interventions into synaptic transmission are a widely used principle of the pharmacology for treatment of neurological and neuromuscular diseases. Thus drugs that influence the corresponding uptake of transporters into synaptic vesicles or that influence re-uptake of the secreted neurotransmitter from the synaptic cleft into the cell are employed. Uptake of neurotransmitters into the synaptic vesicles takes place via 15 transporters located in the membrane thereof, which in general are coupled with and driven by the electrochemical gradients for protons across the vesicle membrane (cf. publications (81) and (82)). If these gradients are changed, the uptake of transmitters are modified, in some cases differentially. On the one hand, particular CLC channels are possibly present only in particular subpopulations of nerve cells or synaptic vesicles (e.g. 20 for particular neurotransmitters), and on the other hand the electrochemical gradient for protons consists of two components ( $\Delta\text{pH}$  and  $\Delta\psi$ ), to which the various transporters are coupled. The uptake of acetylcholine is thus chiefly driven via the pH gradient, while the uptake of glutamate is chiefly driven by the electrical potential  $\Delta\psi$ .

The electrochemical gradient for protons in synaptic vesicles is built up by the 25 proton pump in conjunction with chloride channels. The presence of a chloride conductivity reduces the electrical component  $\Delta\psi$  of the gradient and increases the  $\Delta\text{pH}$  component. Inhibition of a chloride channel in synaptic vesicles thus reduces  $\Delta\text{pH}$ , but increases  $\Delta\psi$ ; as a result e.g. the uptake of acetylcholine is reduced but the uptake of glutamate is increased, provided that the channel occurs on both vesicle types. 30 According to a particular embodiment, conversely, a specific stimulation of individual chloride channels is also envisaged. This would result e.g. in a reduction in glutamate uptake and an increase in acetylcholine uptake.

In addition to the possibility of modulating the concentration of neurotransmitters 35 in synaptic vesicles, such substances can also influence the transport of the vesicles within the cell, e.g. also endo- and exocytosis. This follows from the observation that in the event of CIC-5 KO, endocytotic trafficking is greatly reduced (cf. (8)), and from the fact that the endocytotic and exocytotic pathway can be impaired considerably by reducing the vesicular pH (cf. publications (69) to (74)).

Substances, which influence and in particular inhibit, the activity of the CIC chloride channels CIC-3, CIC-4, CIC-6 and/or CIC-7, which occur predominantly intracellularly, are thus suitable as therapeutic agents for neurological and neuromuscular diseases and other nerve diseases and, in the case of CIC-7, for osteoporosis, Paget's 5 disease, and other bone-degrading diseases. In the context of the invention, these channel proteins are therefore useful as molecular targets in order to discover and develop substances for the treatment of such diseases.

Several assays are suitable as methods for discovering and testing such substances. According to one embodiment of the invention, binding of the substances to 10 the target molecule is tested by methods well-known to the skilled person. For this, the channel proteins are expressed endogenously, e.g. by osteoclasts, or heterologously, e.g. by bacteria, yeasts or mammalian cells, and purified. Appropriate processes are well-known to the skilled person. In a preferred embodiment of the invention binding of the substances to the proteins can be investigated by well-known methods of fluorescence 15 correlation spectroscopy and fluorescence intensity distribution analysis (cf. references (85) to (88)). In another preferred embodiment, the measurement is carried out by similarly well-known methods of plasmon resonance measurement (cf. references (89) to (94)). In yet another embodiment, the binding of ligands to the CIC channels CIC-3, CIC- 4, CIC-6 and/or CIC-7 is measured by the use of labeled ligands, the label being 20 radioactive, fluorescent or any other label that can be identified specifically when compared the same un-labelled ligand. Methods are well-known to the person skilled in the art.

Another preferred method of this invention for the identification of substances which act on the chloride channels CIC-3, CIC-4, CIC-6 and/or CIC-7 comprises test 25 systems in which the corresponding channel protein is expressed functionally, either endogenously or heterologously. Such systems are also appropriate for testing the substances found in the above binding processes for functional effects. In this case, measurement is carried out via the function of the channel protein, which changes either currents, potentials or pH values in particular systems, which then either are measured 30 directly, or their effect on the detection systems is measured. In another preferred method of this invention measurements as above are not performed on the wild-type form of the channel, but on a mutant channel protein that may for instance reside in a different compartment (e.g. plasma membrane) that is easier to study.

In another preferred method of this invention, the structure of CIC-3, CIC-4, CIC- 35 6, and/or CIC-7 may be used to identify or optimize substances binding to the channel protein by molecular modeling.

Another preferred method of this invention for the identification of substances which act on the chloride channels CIC-3, CIC-4, CIC-6 and/or CIC-7 comprises test systems in which the corresponding channel protein is expressed functionally, but the

other channel proteins are either present to a lesser degree or are not present at all. Such systems are also appropriate for testing the substances found in the above binding processes for functional effects. In this case, measurement is carried out via the function of the channel protein, which changes either currents, potentials or pH values in particular systems, which then either are measured directly, or their effect on the detection systems is measured.

In a preferred embodiment of the invention, the activity of the substance on a channel CIC-x (x = 3, 4, 6 or 7) is measured on cells or preparations derived therefrom (such as membrane preparations or vesicles) which exclusively or preferentially 10 (predominantly) express only the channel CIC-x (e.g. CIC-7 in the search for osteoporosis medicaments).

The specificity is tested by measuring the activity of test substances, for example, on cells which exclusively or preferentially (predominantly) express only the CIC-7 channel. These cells or cell lines are obtained, for example, by isolation of the 15 germ cells and somatic cells, which contain nucleic acid sequences encoding chloride channels, from non-human mammals, preferably rodents (in particular mice). In these cells as many as possible of the chloride channel encoding nucleic acid genes— with the exception of the chloride channel for which a specific inhibitor is being searched for — are modified by mutation, truncation, complete deletion and/or partial deletion such that the 20 particular chloride channels are not expressed or are expressed non-functionally. Non-functionally in this context means that the chloride channel protein is expressed such that the transport function of the chloride channel is reduced or suppressed completely. These genetic or genetically engineered modifications are also called knock-out. Corresponding genetically modified mice (as an example of a non-human mammal) are 25 also called knock-out mice or KO mice. Natural mutants, for example, exist as knock-outs of the CIC-1 channel in mice and humans, i.e. a non-functional or absent expression of the CIC-1 channel occurs naturally in a certain percentage of the population, which leads to myotonia congenita. In the case of a knock-out of the CIC-K1 channel or of the CIC-KB channel, diabetes insipidus (in mice) or Bartter's syndrome (in humans) occurs. A knock- 30 out of CIC-5 in humans leads to Dent's disease.

The specificity of substances binding to or modifying CIC channels can of course also be measured by other methods, for example on the isolated channel protein, which can be obtained e.g. by over-expression. Appropriate processes for cloning and expression of the nucleic acid sequence encoding the corresponding channel protein are 35 known in the greatest of detail to the skilled person. The specificity can furthermore also be determined directly using suitable assays, such as e.g. the "pit assay" (see below), which are well-known to the skilled person.

The experimentally generated knock-out of ion channels and in particular of chloride channels is well-known to the skilled person and is described, for example, in

publications (1) to (8) cited in the appendix. It is furthermore known that numerous modifications of the nucleic acid sequences, which code for chloride channels lead to a lack of function or expression of the proteins (cf. publications (9) to (24)). The general structural build-up and the transmembrane topology of the chloride channels is shown in 5 the diagram in fig. 1. For example, individual point mutations in domains D3 to D5 already lead to disturbances in or a lack of expression or to expression of a protein which has no chloride channel properties. The same effect can be achieved by truncation in the region of domains D10 to D12 or generally by truncation in transmembrane-spanning domains. The gene, i.e. the nucleic acid sequence which codes for the chloride channel, can of 10 course also be deleted completely or replaced by a nucleic acid sequence, which codes for another protein, or the promoter region which controls the gene expression can be mutated. The aim of the genetic modification is to suppress the protein expression or to effect non-functional expression of the protein. Alternatively, a so-called knock-down with 15 which the genetic engineering modifications lead merely to a restriction in the chloride channel function, without completely suppressing the transport properties, is also possible. Such knock-down strategies are well-known to the skilled person and include e.g. antisense strategies or ribozyme strategies, i.e. knock-down using antisense oligonucleotides and ribozymes, but are not limited to these. Methods used in the knock-down are described in more detail in publications (25) to (36) cited in the appendix, to 20 which reference is expressly made here.

In the context of the present invention, it is possible to use both somatic cell lines, which are produced from a genetically modified non-human mammal (rodent, in particular mouse) and those cell lines in which the expression of the corresponding channels CIC<sub>xxx</sub> has subsequently been reduced or abolished by genomic mutations of 25 the somatic cell line and/or the expression of the channels has been down-regulated by other processes, such as e.g. via antisense technology or ribozyme or RNAi strategies. This down-regulation or reduction of expression can in particular also be inducible and, thus, prevent or alleviate problems of cell survival and other problems, which can arise from switching off several chloride channels at the same time. These cell lines can also 30 be of human origin.

The identification and testing of substances for chloride channel-specific actions preferably starts from genetically-modified non-human mammals or from cell lines in which preferably two or three chloride channels are not expressed or are expressed non-functionally. In the case of the chloride channel CIC-7, the expression of this chloride 35 channel should not be impaired, so that a knock-out or knock-down of one or more other chloride channels, for example from the group consisting of CIC-3, CIC-4, CIC-5 and CIC-6, must take place. Corresponding conditions also apply if substances which act specifically on another channel are to be identified and tested. Thus, for a test with respect to CIC-4 e.g. cell lines which express only the CIC-4 channel must be

established. Testing of the substances can be carried out either directly on these cells or on preparations obtained from these cells, such as e.g. vesicles, membrane preparations, in particular synaptic vesicle preparations, or purified proteins. Processes for the isolation of these preparations are well-known to the skilled person.

5 The specificity of the action against a particular CIC chloride channel is tested using on the one hand, as described for discovering the substances, cell lines which chiefly or exclusively contain only this particular chloride channel in the intracellular compartments tested, or using the abovementioned preparations derived therefrom. Substances which show the expected effect in these cell lines or on preparations derived  
10 therefrom are then tested on other cell lines or preparations derived therefrom which do not have this channel. If they are specific, they should have no effect on these cell lines. More specific assays follow, depending on the channel: If e.g. the effect on the CIC-7 channel is to be tested with respect to osteoporosis, cultured wild-type (WT) osteoclasts can be tested in a "pit assay" (cf. publications (52) to (58)) on dentine, ivory, bone or  
15 other suitable substrates and, for example, the formation of holes in the substrate can be investigated or the acidification of the resorption lacunae can be investigated with appropriate dyes (e.g. acridine orange) (cf. publications (59) to (61)).

If inhibition of chloride channels of synaptic vesicles (such as e.g. CIC-3) is aimed at, the acidification of purified synaptic vesicles in suspension can be measured  
20 with dyes in the next step (cf. publications (62) to (64)). An inhibition of the channel should manifest itself in an inhibition of the rate of acidification, and the specificity can be checked by isolating synaptic vesicles from the corresponding KO mouse. The substance should have no effect on the rate of acidification of synaptic vesicles isolated from those mice. In further steps, the specificity for particular types of synaptic vesicles can be tested  
25 by determining the uptake of (e.g. radioactively labelled) neurotransmitters in synaptic vesicles in the presence and absence of the substance. The corresponding methods are well-known to the skilled person (cf. e.g. publications (65) to (68)).

Somatic cell lines can be isolated from various tissues of KO mice, and the material is obtained in a form which is as sterile as possible and is introduced, either in  
30 the native form or preferentially after enzymatic digestion, into appropriate cell culture containers (e.g. dishes) together with nutrient media (e.g. Dulbecco's MEM, preferably at least initially with added antibiotics) and incubated at 37°C and 5% CO<sub>2</sub>. The cells are multiplied with standard techniques of cell culture, according to a particular embodiment of the invention the cell line being immortalized by transfection with appropriate genes  
35 (e.g. SV40 largeT antigen, or telomerase) (cf. publications (37) to (39)). As an alternative, KO mice can be crossed with a mouse strain which expresses an appropriate immortalization gene (such as e.g. the immorto mouse, cf. publications (40) to (42), and also other mice (cf. publication (43)) which express these genes, possibly under the control of an inducible promoter).

In particular, these cell lines can be further developed as a test system by transfecting them with appropriate constructs which express proteins which serve directly or indirectly as an indicator for the measurement method. For example, it is possible to express chimaeric proteins which, on the basis of particular protein sequence signals, are 5 diverted specifically into particular compartments. The other part of the chimaeric unit contains either an appropriate indicator protein directly, such as e.g. pH-sensitive fluorescent proteins, such as particular GFP mutants (cf. publications (44) to (46)), or binding sites for diverting indicator substances, such as e.g. antibodies (cf. publication (49)) or biotin-coupled dyes (cf. publication (47)) into these compartments (cf. (44) to 10 (51)).

A particular embodiment of the invention thus relates to the use of the abovementioned non-human mammals or somatic cell lines (of human or non-human origin) for identification of substances which act on synaptic transmission. A test system and a process are provided for the first time for the identification and testing of 15 substances which inhibit or otherwise influence in activity (i.e. for example activate or modify its/their regulation) the chloride channel CIC-3, CIC-4, CIC-6 and/or CIC-7, in particular substances which are suitable for treatment of neurological and neuromuscular diseases and other nerve diseases or as psychotropic pharmaceuticals.

Compounds that may be useful for the treatment of oestoporosis may be 20 distinguished from other compounds by their effectiveness in influencing CIC-7. That is to say, for example, active compounds against CIC-3 (which occurs on synaptic vesicles) will not interfere with the extracellular acidification of osteoclasts. On the other hand, substances which act on CIC-7 can optionally be modified such that they cannot cross the blood-brain barrier and therefore cannot act in the CNS. Such methods are well- 25 known to the skilled person. It is also conceivable that substances are diverted (directed or sorted) specifically into certain neurone groups (e.g. by binding to specific surface receptors), or the substances are first metabolized to the active substance via specific enzymatic activities present in specific subsets of neurones.

A test of the specificity can be carried out, as mentioned above, on the one hand 30 with the aid of appropriate cell lines which express only particular channel types, on preparations derived therefrom (see above) or in specific test systems, such as osteoclasts in culture, or on synaptic vesicle preparations from WT and KO animals.

The present invention relates in particular to a nucleic acid sequence which codes for a protein from the group consisting of the chloride channels CIC-3, CIC-4, CIC- 35 6 and CIC-7, wherein the nucleic acid sequence is modified by mutation, truncation or complete or partial deletion.

The invention furthermore relates to a genetically modified non-human mammal, the gametes and somatic cells of which contain nucleic acid sequences which code for a protein from the group consisting of the chloride channels CIC-1, CIC-2, CIC-Ka, CIC-Kb,

CIC-3, CIC-4, CIC-5, CIC-6 and/or CIC-7, wherein the nucleic acid sequence(s) which code(s) for CIC-3, CIC-4, CIC-6 and/or CIC-7 is (are) modified (with respect to the naturally occurring nucleic acid sequence) by mutation, truncation and/or complete or partial deletion.

5 According to a preferred embodiment of the invention the genetically modified, non-human mammal additionally contains the nucleic acid sequence(s) which code(s) for CIC-1, CIC-2, CIC-Ka, CIC-Kb and/or CIC-5 and which is (are) modified by mutation, truncation and/or complete or partial deletion.

According to the invention, somatic cell lines which do not express one or more 10 chloride channels from the group consisting of CIC-1, CIC-2, CIC-Ka, CIC-Kb, CIC-3, CIC-4, CIC-5, CIC-6 and CIC-7 are established or derived from the mammal, which in particular is a rodent and particularly preferably a mouse. As already described, it is also possible to derive from these non-human mammals preparations, such as e.g. vesicle and other membrane preparations, in particular also synaptic vesicle preparations, which 15 have an appropriate expression pattern in respect of the chloride channels and are therefore just as suitable as the KO animals or cell lines as such.

The invention furthermore relates to somatic cell lines in which either the expression of the chloride channels CIC-1, CIC-2, CIC-Ka, CIC-Kb, CIC-3, CIC-4, CIC-5, CIC-6 and CIC-7, in particular from the group consisting of CIC-3, CIC-4, CIC-6 and CIC- 20 7, is reduced by genomic mutations of the somatic cell line and/or the expression of the channels is down-regulated by other processes, such as e.g. via antisense technology or ribozyme strategies. This down-regulation can in particular also be inducible in order to prevent or alleviate problems with the vitality of the cells and other problems which can arise by switching off several chloride channels at the same time.

25 The abovementioned cell lines can also be of human origin.

The invention furthermore relates to the use of a genetically modified, non-human mammal, the germ cells and somatic cells of which contain nucleic acid sequences which code for a protein from the group consisting of the chloride channels CIC-1, CIC-2, CIC-Ka, CIC-Kb, CIC-3, CIC-4, CIC-5, CIC-6 and/or CIC-7, wherein one or 30 more of these nucleic acid sequences is/are modified (with respect to the naturally occurring nucleic acid sequence) by mutation, truncation and/or complete or partial deletion, for the identification and testing of substances which are suitable for inhibiting one or more chloride channels.

Mammals in which one or more of the nucleic acid sequences which code for 35 proteins from the group consisting of the chloride channels CIC-1, CIC-2, CIC-Ka, CIC-Kb, CIC-3, CIC-4, CIC-5, CIC-6 and CIC-7 is modified (with respect to the naturally occurring nucleic acid sequence) by mutation, truncation and/or complete or partial deletion, in each case one of the sequences which code for CIC-7, CIC-3, CIC-4 or CIC-6 not being modified, so that this chloride channel is expressed normally, i.e. functionally.

Instead of mammals, the abovementioned cell lines (human and non-human) or preparations derived therefrom (see above) can also be used.

Finally, the invention relates to a process for the identification and testing of substances which are suitable for inhibiting or otherwise influencing in its/their activity, i.e. 5 for example activating or modifying their regulation, one or more chloride channels from the group consisting of CIC-3, CIC-4, CIC-6 and CIC-7. In this process, on cell lines or cells (or preparations derived therefrom, in particular membrane preparations, such as vesicles; see above) which express only one chloride channel from the group consisting of CIC-3, CIC-4, CIC-6 and CIC-7, the luminal pH of the compartments which express the 10 channel and/or the potential across the membrane enclosing the channel is determined. These cell lines or cells are then brought into contact with the substances to be tested, and the luminal pH of the compartment, which expresses the channel and/or the potential across the membrane enclosing the channel is determined again. A change in one or both of the physical parameters means that the test substance influences the chloride 15 channel in question. An increase in the pH means that it is a substance which (partially) hinders or (partially) inhibits the chloride channel. Lowering of the pH indicates an acidification of the compartment and therefore a substance which activates the chloride channel. Measurement of an increase in the potential likewise means that it is a substance which (partly) hinders or (partly) inhibits the chloride channel. Lowering of the 20 potential indicates a substance which activates the chloride channel. The activity of a substance in respect of its ability to influence the chloride channel in question is higher the lower the concentration of substance that has to be added in order to effect a change in the physical parameter or parameters.

The process according to the invention is based on the principle that a change in 25 the activity of intracellular chloride channels can change the luminal pH of the compartments which express them and/or the potential across the membrane enclosing them. Chloride channels allow a charge compensation for the proton pumps occurring in the same vesicles (e.g. of the endo- or exocytotic pathway), which has the effect of a higher pump output and therefore a higher acidification of the compartment. At the same 30 time, they lower the electrical potential across this membrane. An inhibition or switching off of the chloride channels would therefore result in a reduced acidification and a higher electrical potential, but a stimulation of their activity would result in an increased acidification and a lowering of the electrical potential. In the present invention the effect of the substances to be tested on the corresponding chloride channels is measured 35 indirectly via one or more effects of the changed acidification and/or potential of intracellular compartments. In order to identify specific substances for a particular channel type, in the preferred use the compartments measured should as far as possible contain only one chloride channel against which the test is being carried out. The chloride channel specificity is demonstrated by carrying out control studies on cell lines which

express another channel. If the compartments express more than one chloride channel, further measurements must be carried out on other KO cell lines or mice, as described above.

The process according to the invention for the identification and testing of substances which are suitable for inhibiting one or more chloride channels from the group consisting of CIC-3, CIC-4, CIC-5, CIC-6 and/or CIC-7 is characterized in that

- 10 a) on cells which express only one or chiefly or predominantly only one of the chloride channels mentioned, the luminal pH of the compartments which express the channel and/or the potential across the membrane enclosing the channel is measured,
- 15 b) the cells are brought into contact with a substance and
- 15 c) the luminal pH of the compartments which express the channel and/or the potential across the membrane enclosing the channel is measured again on the cells,

the difference between the pH and/or the membrane potential before and after addition of the substance determining the activity of the substance.

20 As already mentioned, one process variant consists of direct measurement of the pH of intracellular organelles or measurement of the cell effects which occur as a result of the change in pH. Several methods are possible for measurement of the pH. There are, for example, dyes of which the fluorescence is pH-dependent or which concentrate selectively in compartments with particular pH values when the cells are incubated with 25 them or their precursor stages (examples: acridine orange, Lysotracker and other dye from Molecular Probes, Eugene, Oregon, USA). A higher specificity for particular compartments can be achieved e.g. by endocytotic uptake of dyes (staining of endocytotic compartments, depending on the uptake time, early or late up to lysosomal compartments; cf. e.g. publications (71), (75), (76)). An even higher specificity for 30 particular compartments can be achieved by binding the dyes via coupled molecular groups (such as e.g. specific antibodies or biotin) to particular target molecules, which occur in an increased amount or exclusively in particular compartments, i.e. which are expressed in the cell line (e.g. by permanent transfection), the cell line being brought into contact with the dye. These target molecules can also be prepared via molecular cell 35 biology techniques, e.g. by fusing a target control signal, which directs the molecule into a particular compartment via an appropriate machinery of the cell, to a corresponding binding motif (e.g. epitope for antibodies or avidin) by a molecular biology method and then expressing the construct in the corresponding cell line used for testing the substances. Using such techniques, it is possible to examine and measure predominantly 40 or even very specifically only those compartments that contain the channel of interest. The accumulation of particular substances in compartments with a particular pH (such as

e.g. acridine orange or Lysotracker from Molecular Probes) or indirect tests in which pH-dependent reactions in the compartments are utilized to produce indicator substances (e.g. by pH-dependent proteolytic cleavage), which are then easy to detect (e.g. using dyes (cf. publications (95) and (96)), although the detection is not limited thereto), are 5 also used e.g. as a further technique for pH measurement.

Alternatively or in addition, the membrane potential in these compartments can be measured, e.g. via potential-sensitive dyes (cf. e.g. publication (67)). Alternatively, protein-coded potential sensors (cf. e.g. publication (77)), which are possibly also diverted or sorted specifically, are also possible here.

10 The present invention provides for the first time a test system with which active compounds which are suitable for the preparation of medicaments for treatment of osteoporosis or Paget's disease or for the preparation of medicaments for treatment of neurological and neuromuscular diseases and other nerve diseases or for the preparation of psychotropic pharmaceuticals can be identified and tested. The invention thus also 15 relates to the use of substances, which completely or partly inhibit the chloride channel CIC-7 for the preparation of medicaments for treatment of osteoporosis or Paget's disease, and to the use of substances which completely or partly inhibit the chloride channel CIC-3, CIC-4, CIC-6 and/or CIC-7 for the preparation of medicaments for treatment of neurological and neuromuscular diseases and other nerve diseases or of 20 psychotropic pharmaceuticals. The invention furthermore relates to pharmaceutical compositions (medicaments) for treatment of osteoporosis or Paget's disease which comprise one or more substances which completely or partly inhibit the chloride channel CIC-7, and to medicaments for treatment of neurological and neuromuscular diseases and other nerve diseases and psychotropic pharmaceuticals which comprise one or more 25 substances which completely or partly inhibit the chloride channel CIC-3, CIC-4, CIC-6 and/or CIC-7. The medicaments comprise the active compounds in a formulation suitable for oral or intravenous administration, optionally together with pharmaceutically tolerated carrier substances.

30 The invention is explained in more detail below with the aid of examples.

### Description of the figures

Fig. 1: Transmembrane topology model of CLC channels according to the biochemical study of Schmidt-Rose and Jentsch (T. Schmidt-Rose and T.J. Jentsch, Proc. Natl. 35 Acad. Sci. USA 94 (1997) 7633-7638). N- and C-termini are located intracellularly. Initial hydropathy analysis of CIC-0 showed the presence of up to 13 transmembrane domains (D1-D13). Apart from some prokaryotic CICs, all known CIC proteins have two CBS domains (cf. A. Bateman, Trends Biochem. Sci. 22 (1997) 12-13 and C.P. Ponting, Mol. Med. 75 (1997) 160-163) on the C terminus.

## EXAMPLES

### Example 1

#### Protocol for generating knock-outs by the example of CIC-7

5 The CIC-7 knock-out mouse was produced by standard methods which are well-known to the skilled person and are described in detail, *inter alia*, in method books (cf. e.g. publication (78)). This technique requires several steps. In the first step a DNA construct which, in addition to the target sequence (in this case the genomic sequence of the mouse which contains the gene which codes for the CIC-7 channel), contains 10 appropriate selection markers. In the second step an allele of the target gene is modified in pluripotent embryonal stem cells of the mouse by homologous recombination with the aid of this DNA construct such that it can no longer code for a functional protein. In the third step these recombinant embryonal stem cells are injected into mouse blastocysts, which are then transplanted into the uterus of pseudo-pregnant foster mothers (mice). 15 These then give birth to descendants which are chimaeric, i.e. in addition to the genetically modified cells which originate from the stem cells injected, also contain normal, genetically non-manipulated cells of the blastocysts injected. These chimaeric mice are paired with normal "wild-type" (WT) mice. The descendants are tested (e.g. by southern blotting or by PCR techniques) as to whether the genetically modified, that is to 20 say functionally destroyed, gene has been inherited via the germ line: In the positive case these are now heterozygotic animals in which the channel gene on one of the two chromosomes is destroyed. Heterozygotic mice are crossed with one another to finally obtain homozygotic knock-out animals in which the corresponding channel genes are destroyed on both chromosomes.

25 In the case of the CIC-7 KO mouse, the construct was prepared as follows: With the aid of a rat cDNA probe (protein sequence published in reference (79)); Accession No. for protein and cDNA: Z67744 GenBank), a commercially obtainable genomic phage library in the vector  $\lambda$ FIXII of the mouse strain 129/Sv (Stratagene, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA) was scanned by standard methods of plating out, 30 stripping off filters and hybridization with the radioactively labelled cDNA probe. Several phage clones which hybridized under high stringency were isolated, purified and analysed with standard methods of restriction mapping, partial sequencing and amplification of selected fragments by the PCR. A genomic clone which contained a piece approximately 14 kb in size of the genomic sequence of CIC-7, including the exon 2 (cf. (80)), was 35 selected for preparation of the construct. For this, the genomic clone was digested with the two restriction enzymes BglII and BsrGI, as a result of which the part of the sequence which contains the coding exons 3, 4, 5, 6 and 7 was removed. This part was replaced with a DNA fragment approximately 1.6 kb in size which contains a neomycin resistance cassette driven by the phosphoglycerate promoter, by ligating the cassette into the

genomic sequence by appropriate enzymatic reactions using standard processes. After transformation of bacteria with the corresponding vector containing this construct, bacterial colonies containing the correct construct were isolated, the DNA was extracted, and after digestion with HindIII, a thymidine kinase cassette was attached at the 5' end of 5 the construct for negative selection. After renewed transformation, isolation and checking of the now finished construct, pluripotent embryonal stem cells (mouse) were transfected with it by electroporation and plated out and multiplied under appropriate culture conditions (culture on "feeder layers" in the presence of leukaemia inhibitory factor (LIF) to prevent differentiation). The cells were selected with G418 (selection for the presence 10 of the neomycin resistance cassette) and gancyclovir (selection for the absence of the thymidine kinase cassette). Resistant clones were isolated, drawn out and analysed by southern blot analysis for homologous recombination on the CIC-7 locus. Correct clones in which the CIC-7 gene on one chromosome was destroyed (i.e. exons 3-7 were replaced by the neomycin cassette), were expanded by growing and injected into mouse 15 blastocysts, as described above, under microscopic control with micromanipulators. The subsequent procedure was as described above. Since the genetic modification was inherited via the germ line, a CIC-7 KO mouse was produced in this way. The absence of the CIC-7 channel protein was demonstrated with the aid of a specific antibody established against an amino-terminal peptide of CIC-7. The CIC-7 KO mouse 20 unexpectedly showed the phenotype of a potent osteopetrosis, accompanied by a retina degeneration and signs of degenerative changes in the central nervous system.

### Example 2

#### Generation of a CIC-3 KO mouse

25 In a manner similar to that described in example 1, a CIC-3 KO mouse in which exon 3, which codes for sequences in the first transmembrane domain (cf. publications (83) and (84)), was deleted was also produced. This construct furthermore leads to a premature stop in translation, so that a very small, truncated protein was predicted. CIC-3 protein was no longer to be detected in the KO mouse with a specific antibody 30 established against an amino-terminal peptide of CIC-3. The CIC-3 KO mouse showed a degeneration of the hippocampus and a degeneration of the retina. It was possible to demonstrate that the CIC-3 chloride channel occurs in intracellular, predominantly endosomal compartments and synaptic vesicles. pH measurements showed that the absence of CIC-3 caused a reduction in the acidification of synaptic vesicles.

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